FEDERAL REPUBLIC OF GERMANY

GERMAN PATENT AND TRADE-MARKS OFFICE

12 **Unexamined Patent Application**

10 **DE 100 49 363 A1**

51 Int. Cl. 7: **C 12 Q 1/68**

21 Application no.: 100 49 363.7
22 Date of filing: 5.10.2000
43 Date of publication: 31.10.2001

66 Internal priority:
100 19 553.9 20.04.2000

74 Agent:
PFENNING MEINIG & PARTNER GbR, 80336 Munich

72 Inventor:
Wehmeier, Lutz, Dr., 30900 Wedemark, DE; Tamak, Cengiz, Dr., 31275 Lehrte, DE; Waschütze, Stefanie, Dr., 29227 Celle, DE

56 Patents cited:
US 59 72 602 A
US 56 41 658 A
Databank MEDLINE at STN, AN 93341588 on: Prenatal determination of fetal RhD type by DNA amplification. BENNETT, P:R: et al., NEW ENGLAND JOURNAL OF MEDICINE, (1993 August 26) 329(9) 607-10 [searched on 28.05.01] ;
Databank MEDLINE at STN, AN1999056309 on: Geno-typing of RHD by multiplex polymerase chain reaction analysis of six RHD-specific exons. MAASKANT-VAN WIJK P.A. et al., TRANSFUSION (1998 Nov.-Dec.) 38 (11-12) 1015-21 [searched on 28.05.01]; Internet document, address www.bloodjournal.org./cgi/content/abstract/85/10/2975, of the abstract of the publication "Rapid RhD geno-typing by polymerase chain reaction-based amplification of DNA", SIMSEK, S. et al., Blood (1995) 85(10) 2975-2980 [searched on 28.05.01];
Internet document, address www.bloodjournal.org/cgi/content/full/89/7/2568, of the publication "Evidence of genetic diversity underlying RhD, weak D (D"), and partial D phenotypes as determined by multiplex polymerase chain reaction analysis of the RHD gene". AVENT, N.D.et al., Blood (1997) 89(7) 2568-2577 [from 28.05.01];
Internet document on Fig. 1 of (4), address www.bloodjournal.org/cgi/content/full/89/7/2568/F1 [searched on 28.05.01];

The following information is gathered from the documents filed by the applicant

Request for examination made according to § 44 of the Patent Act

54 Method, diagnostic kit and microarray for determining the rhesus factor

57 The present invention relates to a method, a diagnostic kit, a microarray (for example, DNA chip) as well as their use for the determination of the rhesus factor of a human, in particular a human fetus for use, for example, in the field of medicine, in particular in prenatal diagnosis.
The diagnostic kit according to the invention, comprises at least a pair of oligonucleotides (reverse primer, forward primer) in which case the two oligonucleotides of the pair are suited as primer for the amplification by means of polymerase chain reaction of in each case one of the two complementary strands of a DNA segment of the human RhD gene.

[0001] The present invention relates to a method, a diagnostic kit, a microarray (for example, DNA chip) as well as their use for the determination of the rhesus factor of a human, in particular of a human fetus. Such a method and diagnostic kit are used in the field of medicine, in particular in prenatal diagnosis.

[0002] The object of such a prenatal diagnosis is to determine reliably the rhesus factor of a human, in particular of a fetus.

[0003] The rhesus factor is of great clinical importance, in particular for hemolytic diseases of newborns, incompatibility reactions during transfusions and certain hemolytic autoimmune diseases. The antigens of the rhesus factor system are expressed (sic) on (sic) polypeptides which are expressed (sic) by two highly homologous genes RhD and RhCE. The RhD antigen determines whether a human is rhesus positive or rhesus negative. Various RhD variants occur because of gene conversion between RhD and RhCE or through mutations in the RhD gene which brings about an amino acid substitution.

[0004] According to the prior art, serological methods are available for the determination of the rhesus factor.

[0005] The object of the present invention therefore is to provide a diagnostic kit, a microarray, a method as well as uses thereof, with which a definite and reliable determination of the rhesus factor of a human is possible.

[0006] This problem is solved through the diagnostic kit according to claim 1, the microarray according to claim 21, the method according to claim 24 as well as the use according to claim 42. Advantageous further developments of the diagnostic kit according to the invention, the method according to the invention and the use according to the invention, are given in the respective dependent claims.

[0007] The diagnostic kit according to the invention, comprises at least a pair of oligonucleotides which are required as reverse primer and forward primer in the amplification by means of polymerase chain reaction. Such oligonucleotide pairs also are used in the method according to the invention. The oligonucleotides of this pair are selected here in such a way that when using these oligonucleotides in a polymerase chain reaction, a DNA segment of a human DNA is amplified, which is part of the DNA of the human RhD gene. If an amplification of a DNA segment takes place in a polymerase chain reaction carried out in such a way, it can easily be determined whether or not a RhD gene is present in the examined DNA substance. Consequently, it can be determined in all probability whether the human is rhesus positive or rhesus negative.

[0008] It is advantageous if, for the amplification, six oligonucleotide pairs are used and included in the kit respectively by means of which six amplificates are developed in rhesus positive individuals while in rhesus negative individuals, none of the fragments is produced. With suitable selection of the oligoneucleotide pairs the kit and method according to the invention, allows (sic) further the determination of rhesus subtypes

which come about through the loss of one or several RhD exons (RhD/RhCE gene conversion of these regions and distinguish themselves serologically by a weaker immunogeniety.

[0009] Thus, advantageously the method uses and the kit includes respectively several pairs of oligonucleotides which, for example, in each case amplify a segment of various exons (coding subregions) of the RhD gene. Various subgroups of the rhesus factor thus also can be established by determining generally by means of a multiplex polymerase chain reaction not only the presence of the RhD gene, but also possible changes in individual exons of this RhD gene. Advantageously, the oligonucleotides of a pair have in each case one of the following sequence pairs:
TCGGTGCTGATCTCAGTGGA and ACTGATGACCATCCTCATGT
or
CACATGAACATGATGCACA and CAAACTGGGTATCGTTGCTG
or
GTGGATGTTCTGGCCAAGTT and CACCTTGCTGATCTTACC
or
GTGGCTGGGCTGATCTACG and TGTCTAGTTTCTTACCGGCAAGT
or
AGCTCCATCATGGGCTACAA and ATTGCCGGCTCCGACGGTATC
or
AACAGGTTTGCTCCTAAATATT
and
AAACTTGGTCATCAAAATATTTAACCT

[0010] If the relevant exons of the RhD gene are present, these oligonucleotide pairs lead to the amplification of the exons 3, 4, 5, 6, 7 and of the exon 9 respectively of the RhD gene. For easier differentiation of the individual amplification products an oligonucleotide of a primer pair can be labeled in each case by means of a fluorophore so that an analysis is possible, for example, by means of ABI Genetic Analyzer™ of the firm PE Biosystems™.

[0011] Advantageously the diagnostic kit contains moreover the substances, required for carrying out a polymerase chain reaction, as are offered already by various producers.

[0012] The determination of the presence and of the allelic variability respectively of the RhD gene can be carried out by means of a microarray, for example, DNA chip in which case the individual wells of the chip have oligonucleotides which specifically hybridize with certain segments of the RhD gene, for example, of the exon 3 to 7 and/or 9. For the design and the function of an oligonucleotide microarray reference generally is made, for example, to EP 0 373 203. The determination can take place in this case with or without amplification of the looked for DNA segment and without or after a suitable restriction digestion of the looked for DNA segment.

[0013] The method according to the invention, the nucleic acid chip according to the invention and in particular the diagnostic kit according to the invention, has (sic) the big

advantage that any pathologist and any medical laboratory as well as any scientific laboratory which might carry out such investigations gets (sic) at their disposal the essential or all substances adapted to one another in one single diagnostic kit, if required, so that the expensive or any preparatory work for the laboratory is eliminated and the rhesus factor of a human, in particular a fetus, can be determined in a simple manner. In particular the primer oligonucleotides are developed and already tested so that the main work in the development of relevant amplification procedures is eliminated. Because the selection of suitable oligonucleotides which then in practice in fact also lead to the desired, reliable result, is in no way trivial and not easy to carry out for a medical or scientific laboratory.

[0014] With the proposed method, the microarray and diagnostic kit it is in particular possible for the first time to determine in a simple and cost effective way the rhesus factor of a fetus from the blood of the mother to a large extent non-invasively and on the spot. For this the individual pathologist or the individual medical laboratory then only requires in addition a table centrifuge, a conventional thermocycler (PCR device) as well as, if needed, a device for separating the individual DNA fragments. However, such devices are already available in many medical laboratories. This, for example, can be a gel electrophoresis device or a capillary electrophoresis device.

[0015] Such a conventional capillary electrophoresis device is sold, for example, by PE Biosystems™ under the name PE ABI Genetic Analyzer 310™. Relevant thermocyclers for the amplification of the DNA are sold, for example, by the firm PE Biosystems™ under the name Gene Amp 2400™ or also Gene Amp 9700™.

[0016] According to the invention, the diagnostic kit can also be further developed to the effect that to the diagnostic kit not only oligonucleotides for the determination of the rhesus factor of the fetus, as described above, are added but also other pairs of oligonucleotides for other determinations. Accordingly, in other wells of the chip, relevant oligonucleotides are arranged which hybridize DNA segments of other to-be-determined genes.

[0017] In this case, it is possible for the user to carry out in a simple way not only a determination of the rhesus factor of the fetus, but at the same time also other molecular biological tests.

[0018] According to the invention, the method, the microarray and the diagnostic kit for the determination of the fetal rhesus factor is used by first taking a maternal blood sample. This maternal blood sample now can be processed further in various ways.

[0019] Firstly, the DNA containing constituents of the maternal blood sample subsequently is concentrated. This, for example, takes place through blood sedimentation, if needed, supported through centrifugation for the acceleration of the sedimentation of the cellular constituents. By means of this step the cell fraction in a blood sediment is concentrated which is suitable for the following DNA isolation.

[0020] The blood sediment is taken and a lysis of the maternal erythrocytes as well as the nucleated fetal erythrocytes is carried out by means of which the DNA from the fetal erythrocytes is released.

[0021] Thereafter a high speed centrifugation takes place, for example, at 50,000 g for 30 minutes which leads to pelleting of cells of the mother and of the fetus (for example, lymphocytes), the DNA of the fetus released from the fetal erythrocytes as well as cell-free DNA from the fetus originating from various cell types.

[0022] Thereupon the pellet is taken and a lysis of the lymphocytes of both maternal and fetal origin takes place. After precipitation of the proteins, the proteins are centrifuged off. In the supernatant there is then free DNA of the mother and of the fetus. This is precipitated with isopropanol and subsequently centrifuged off. The pellet then contains the DNA of the mother and fetus and is subsequently rehydrated.

[0023] Secondly, for the extraction of the to-be-identified DNA the plasma/serum of the maternal blood sample can also be used. For this, the cell fraction of the mother and of the fetus is, if required, first allowed to settle by means of blood sedimentation or separated through centrifuging. The supernatant then contains cell-free DNA, in particular cell-free fetal DNA. This cell-free DNA from the plasma/serum can now be separated directly. With this method the concentration step for the cells of the first variant is avoided. Then a concentration or separation of the DNA of mother and fetus contained in the plasma/serum is not required, since in the plasma/serum about 800 times more fetal DNA is present than fetal DNA from fetal cells which circulate in the maternal blood. The amount of fetal DNA of all the DNA contained in the plasma is between about 3% and 7%. Here, the cell-free fetal DNA by and large increases steadily during the course of the pregnancy, in the last trimester of the pregnancy even very strongly.

[0024] In a further step this cell-free fetal DNA from the plasma/serum then can be isolated in a conventional way, for example, by means of a commercially obtainable kit and is thus available for the further investigation, for example, through application on the microarray according to the invention.

[0025] The DNA which was recovered from the fetal nucleated cells from the maternal blood sample as well as the DNA from the serum/plasma of the maternal blood sample is, if necessary, subsequently processed further by carrying out a specific amplification of the to-be-determined DNA segments in the DNA contained in the pellet by means of polymerase chain reaction (PCR) or Multiplex-PCR. For this, the diagnostic kit according to the invention, or the therein contained primer pairs according to the invention, are now used.

[0026] Subsequently the amplified DNA is determined.

[0027] On the one hand, this can take place by means of the microarray according to the invention, or by separation by way of gel electrophoresis. Provided that one of the oligonucleotides from a primer pair is labeled with a fluorophore, the identification can

take place by way of the detection of the corresponding fluorescence, for example, in the relevant well of the chip. However, this can, for example, also take place by means of a Genetic Analyzer 310™ of the firm PE Biosystems™. Both methods have a very high sensitivity and reliability.

[0028] The resulting fluorescence data are analyzed and utilized as evidence for the rhesus factor of the fetus.

[0029] In the present method it should be taken into consideration that no separation of the constituents of the maternal blood sample into, for example, a cellular or non-cellular fraction or, for example, into a fraction with exclusively maternal or exclusively fetal constituents is carried out. The method is therefore very simple to carry out.

[0030] The case in which both the mother and the fetus are rhesus positive or the mother as well as the fetus are rhesus negative has medically no complications. The same applies in the case that the mother is rhesus positive and the fetus is rhesus negative. Also in this case no medical complications occur.

[0031] The case is only critical if the mother is rhesus negative and the fetus is rhesus positive. In this case, the danger is that the mother develops antibodies against the rhesus factor of the fetus and, for example, in subsequent pregnancies and in very rare cases already in the current pregnancy, immune reactions occur. However, exactly this case can easily be determined by the present method, since a rhesus positive result of the blood sample, processed as above, in the case of a rhesus negative mother whose rhesus factor was determined serologically in a conventional way at the latest at the start of the pregnancy, clearly indicates a rhesus positive fetus. If in this case, for example, a fluorescence is determined which results from an oligonucleotide belonging to a primer pair that is suitable for the amplification of a DNA segment from a RhD gene, there is inevitably in the case of the fetus a rhesus positive result since according to the assumption, the maternal constituents themselves have no RhD gene. In this case, the conventional prophylaxis can take place. In the case of a rhesus negative mother prophylaxis so far always takes place although it is required only in the case of a rhesus positive fetus. Thus if using the kit according to the invention, a rhesus negative fetus is determined, the up to now usual prophylaxis in the mother can be omitted.

[0032] The method according to the invention, the diagnostic kit according to the invention and their use therefore are suitable for identifying reliably in a simple way the only medically relevant case.

[0033] Moreover, it is possible with the method and kit according to the invention to identify rhesus subtypes which occur due to the loss of one or several RhD exons (RhD/RhCE gene conversion of these regions) and which distinguish themselves serologically by a weaker immunogencity.

[0034] In the following some examples of the method according to the invention, are to be described using the kit according to the invention.

[0035] **Fig.** 1 shows the measurement results of a rhesus positive reference sample;

[0036] **Fig.** 2 the result of a rhesus negative reference sample and

[0037] **Fig.** 3 the result of a multiplex PCR.

[0038] As example of a method according to the invention, a new blood sample was taken from a pregnant woman and taken up in a EDTA buffer, for example, in standardized, commercially available EDTA tubes. Subsequently, 5 to 10 ml of the maternal blood was centrifuged for 20 minutes at 3000 g. The plasma is separated carefully from the pelleted blood constituents and centrifuged again for 20 minutes at 3000 g. Afterwards, the supernatant is transferred to a new vessel. With this procedure the nuclcus-free fetal DNA remains in the plasma. The DNA then is isolated by the standard procedures of the QIAamp Blood Kit™ (Firm Qiagen, Hildcn) and can be used in the following for the determination of the rhesus factor with a primer pair (single PCR) or with several primer pairs simultaneously (Rhesus Factor Multiplex PCR).

[0039] In another example the concentration also can take place by means of a Percoll density gradient centrifugation.

[0040] This method uses the fact that with a constant centrifugal force and medium viscosity the sedimentation rate of particles is proportional to the size of the particle. In the present example, Percoll™ was used as centrifugation medium. This is a silica derivate which as a standard is used for concentrating or separating sub-cellular particles.

[0041First a continuous Percoll gradient is produced which covers a density range of 1.02 – 1.113 g/ml. For this purpose 14 ml of a Percoll NaCl solution with a density of 1.07 g/ml was centrifuged for 30 minutes and at 20,000g. Subsequently, this continuous gradient is layered with 10 ml maternal blood and centrifuged for 5 minutes at 1000 g.

[0042] After this centrifugation step, the maternal thrombocytes are in the serum layer over the gradient and are removed with a Pasteur pipette and discarded. In a further centrifugation step at 1000 g over 5 minutes, the various remaining blood cell types are separated according to their respective densities. The sedimentation layer with the fetal mononuclear erythrocytes occur as bands at a density of 1.09 – 1.10 g/ml and can be removed using a Pasteur pipette. After that, it is washed three times with phosphate buffer saline (PBS, pH = 7.4) and resuspended in 1 ml PBS. The DNA of the mononuclear blood cells then is isolated by the standard procedures of the QIAamp Blood Kit™ (Firm Qiagen, Hilden) and afterwards used for the following single PCR or rhesus factor multiplex PCR.

[0043] As another example for a sample preparation, a blood sample is taken of a pregnant woman and taken up in an EDTA buffer, carried out as above. Subsequently, blood sedimentation is carried out overnight. Alternatively, the sample can also be centrifuged at low g-values. Of this blood sediment, 500 µl is taken up in 900 µl erythrocyte lysis buffer of the Wizzard™ (sic) kit of the firm Promega™. Was thereupon centrifuged in a Sorvall™ centrifuge (rotor SM 24) for 30 minutes at 50,000 g. In this way, the predominantly maternal lymphocytes as well as the DNA, present cellfree after the lysis of the infantile erythrocytes, are pelleted. The DNA now is isolated from the pellet by means of a conventional, commecial DNA isolation kit (for example, Wizzard (sic) Kitz (sic) of the firm Promega™). The extracted DNA is taken up in 20 µl of the dehydration solution according to the respective DNA isolation kits.

[0044] The isolation of fetal nucleus-containing erythrocytes is moreover possible by means of FACS flow-through cytometry. For this purpose, a concentration of all mononuclear blood cells first takes place by means of Percoll density gradient centrifugation (see above). The sedimentation layer with mononuclear blood cells is here removed with a Pasteur pipette, washed three times with phosphate buffer saline (PBS, pH = 7.4) and finally resuspended in 1 ml PBS.

[0045] Subsequent to the density gradient centrifugation, 800 µl of the obtained blood cell suspension is labeled and incubated respectively for 60 minutes at 4°C with a phycoerythrin-conjugated monoclonal antibody against the glycophorine A surface protein (BD-Pharmingen) and a fluorescein isothiocyanate (T9-FITC) conjugated monoclonal antibody against the transferrin receptor (CD36) (BD-PharMingen). A third fluorescence channel is used for the negative discrimination of T, B and NK cells. The end concentration of both antibodies is in each case 0.2 µg per 10^7 cells. The labeled cells are washed twice with PBS and subsequently 10^7 cells are resuspended in 1 ml PBS.

[0046] A FACS Vantage SE flow-through cytometer (Becton Dickinson) is used for the isolation of nuclei-containing erythrocytes. The system is configurated with a water-cooled dual wavelength argon laser (emission wavelength 488 nm and 365 nm UV) and a air-cooled helium neon (HeNe) laser and calibrated with fluorescent beads (Becton Dickinson). The CellQuest software is used for data acquisition, instrument control as well as the statistical analysis. The sorting of the blood cells is carried out at cell rates of 20,000 – 25,000 cells per second in which case the cell size ("forward scatter") and the granularity as well as the surface structure ("side scatter"), the emission of the green fluorescence (Transferrin receptor, T9-FITC), the emission of the orange fluorescence (glycoforin A, KC16-Rd) as well as the emission of the red fluorescence for the other parameters, such as CD45, CD3, CD19 and CD16/56 (labeled APC or Cy5). In order to ensure an additional discrimination of nucleus-containing cells, the Hoechst 33342 DNA stain is used. The excitation takes place at the same time with the UV line of the Enterprise laser. The sorted cells are tranferred directly into a 1.5ml reaction vessel, which is filled with 1 ml PBS, and can be stored at -20°C.

[0047] The fetal DNA is not separated completely from the maternal DNA with the proposed method. However, provided the mother is rhesus negative, a positive RhD

identification always points to a rhesus positive fetus. After the isolation of the DNA a multiplex PCR is carried out.

[0048] For this, the following primers were used in which case a primer from a primer pair is labeled with a fluorescent dye. The relevant primers are given in the following table.

Table 1

Primer name	Sequence	Labeling	Size

[0049] Here the name of the primer is given in the first column of the table. Moreover, there the localization of the to-be-amplified DNA segment is given (exon 3 to 7, 9). In the second column of the table the relevant primer sequence is given, in the third column the fluorescent dye with which the respective primer is labeled. Here, the designation 5'-NED stands for the fluorophore NED™ of the firm PE Biosystems, the designation 5'-HEX for the dye 4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein and the designation 5'-6-FAM for the dye carboxyfluorescein.

[0050] In the last column, the size of the amplification product is given which forms in a PCR with the respective primer pair. As can be seen two different groups of amplification products result with lengths between 52 and 91 base pairs or lengths between 108 and 153 base pairs. Since these are sufficiently far from one another, one of the dyes can be used in each of these groups and a reliable differentiation of the individual amplification products still can be achieved.

[0051] In the present example the PCR reactions were carried out with a PCR block cycler PE 9700 of the firm Applied Biosystems.

[0052] Here, the following mixture is used as reaction starting material in the case of which 100 µl reaction volume was used in each case:

	Volume	End concentration
Puffer = buffer		

[0053] The PCR was carried out in this case with the following thermocycle:

[0054] For the analysis of the PCR results by means of ABI fragment analysis the individual PCR starting materials first were used undiluted in the case of which 1 µl was used in each case for the individual analysis.

[0055] The respective results are represented in **Fig.** 1 and 2. **Fig.** 1 shows the results of the PCR on a blood sample determined serologically for Rhesus-positive with in each case different primer pairs according to Table 1. It can be established that in **Fig.** 1A to 1F in each case a strong fluorescence of amplification products with length of 108 base pairs, 120 base pairs, 153 base pairs, 52 base pairs, 91 base pairs and 72 base pairs respectively were observed.

[0056] The signals in the range below 40 base pairs (see in particular **Fig.** 1C) originate presumably from nonspecific amplificates and do not affect adversely the method according to the invention.

[0057] **Fig.** 2 shows the results of a PCR with a blood sample determined serologically as rhesus negative with in each case different primer pairs according to Table 1.

[0058] From **Fig.** 1A to 1F it can be seen immediately that in this blood sample no quantities of amplification products worth mentioning could be determined with the to-be-expected lengths. The serological result consequently is confirmed completely by the method according to the invention.

[0059] **Fig.** 3 shows the results of a multiplex PCR in which all primer pairs according to Table 1 were utilized simultaneously. All PCR reactions were carried out on a PCR block cycler PE 9700 of the firm Applied Biosystems.

[0060] For the PCR the following reaction starting material with a reaction volume of 50µl was used:

	Volume	End concentration

Puffer = buffer

[0061] The PCR cycles were carried out with the following parameters:

[0062] The individual PCR starting materials first were used undiluted in which case 1µl was used in each case for the fragment analysis.

[0063] **Fig.** 3 shows the results of two measurements in which case in **Fig.** 3A first rhesus positive reference blood, as was typified by serological standard methods, was used as PCR template. In **Fig.** 3B the results of a PCR with a PCR template of rhesus negative reference blood can be seen. In both cases, deviating from the previous measurements, the albumin gene was added as standard as well as the following primers:
albumin fw: GCC CTC TGC TAA CAA GTC CTA C
as well as
albumin rv: GCC CTA AIAA AGA AAA TCG CCA ATC.

[0064] The forward primer ("Albumin fw") is here labeled with the fluorescent dye 5'Ned™.

[0065] In this way, a band resulted in the case of 350 base pairs for the amplification products of the albumin gene.

[0066] Moreover, in **Fig.** 3A the respective signals for the amplification products of all 6 primer pairs can be seen from Table 1. Obviously it was rhesus positive blood which was investigated here.

[0067] In Fig.3B, it can be seen that in a corresponding rhesus negative reference sample no fluorescent band was observed except for the albumin band in the case of 350 base pairs. The kit according to the invention, the microarray according to the invention and the method according to the invention, thus also are suitable with multiplex PCR for differentiating between rhesus negative and rhesus positive blood as well as for the determination of individual sub-types.

Claims

1. Diagnostic kit for the determination of the rhesus factor with at least one pair of oligonucleotides (reverse primer, forward primer) in which case the two oligonucleotides of the pair are suited as primer for the amplification by means of polymerase chain reaction of in each case one of the two complementary strands of a DNA segment of the human RhD gene, and in which case the looked for DNA is part of the human RhD gene.

2. Diagnostic kit according to the previous claim, characterized in that it comprises six pairs of oligonucleotides (reverse primer, forward primer) in which case the two oligonucleotides of the respective pairs are suited as primer for the amplification by means of polymerase chain reaction of in each case one of the two complementary strands of various, looked for DNA segments, and in each case the looked for DNA segment are (sic) part of the DNA of the human RhD gene.

3. Diagnostic kit according to one of the previous claims, characterized in that the looked for DNA segments are part of coding subregions of the RhD gene.

4. Diagnostic kit according to one of the previous claims characterized in that each one of the looked for DNA segments are (sic) part of the coding subregions 3, 4, 5, 6, 7 or 9.

5. Diagnostic kit according to one of the previous claims characterized in that it comprises the substances required for carrying out a polymerase chain reaction.

6. Diagnostic kit according to one of the previous claims, characterized in that it comprises as substances required for carrying out a polymerase chain reaction a buffer solution, magnesium chloride, desoxynucleotide triphosphate as well as a thermostable polymerase.

7. Diagnostic kit according to the previous claim characterized in that it comprises as thermostable polymerase a polymerase from thermus aquaticus (Taq polymerase).

8. Diagnostic kit according to one of the previous claims, characterized in that it comprises as positive control a DNA sample with the in each case looked for DNA segment.

9. Diagnostic kit according to one of the previous claims, characterized in that it comprises moreover for control determination a segment of a DNA coding for an albumin as well two oligonucleotides which in each case are suitable as primer for the amplification of at least one segment of in each case one of the two complimentary strands of the DNA coding for the albumin.

10. Diagnostic kit according to one of the previous claims, characterized in that in each case at least one of the two oligonucleotides of a pair of oligonucleeotides is labeled with fluorophores.

11. Diagnostic kit according to the previous claim, characterized inthat the oligonucleotides of various pairs are labeled with various fluorophores.

12. Diagnostic kit according to one of the previous claim, characterized in that the two oligonucleotides of a pair have in pairs the following sequences:
TCGGTGCTGATCTCAGTGGA and ACTGATGACCATCCTCATGT
or
CACATGAACATGATGCACA and CAAACTGGGTATCGTTGCTG
or
GTGGATGTTCTGGCCAAGTT and CACCTTGCTGATCTTACC
or
GTGGCTGGGCTGATCTACG and TGTCTAGTTTCTTACCGGCAAGT
or
AGCTCCATCATGGGCTACAA and ATTGCCGGCTCCGACGGTATC
or
AACAGGTTTGCTCCTAAATATT and
AAACTTGGTCATCAAAATATTTAACCT

13. Diagnostic kit according to the previous claim, characterized in that the oligonucleotides with the following sequence
GTGGATGTTCTGGCCAAGTT or AGCTCCATCATGGGCTACAA
are labeled with fluorophore carboxyfluorescein.

14. Diagnostic kit according to the previous claim, characterized in that the oligonucleotides with the sequence
CACATGAACATGATGCACA or AACAGGTTTGCTCCTAAATATT
are labeled with the fluorophore 4, 7, 2', 4', 5', 7' – hexachloro-6-carboxyfluorescein.

15. Diagnostic kit according to the previous claim, characterized in that the oligonucleotides with the sequence
TCGGTGCTGATCTCAGTGGA or GTGGCTGGGCTGATCTACG
are labeled with the fluorophore NED™ of the firm PE Biosystems.

16. Diagnostic kit according to one of the previous claims, characterized in that it comprises a manual for carrying out the polymerase chain reaction and/or a manual for carrying out a fragment analysis.

17. Diagnostic kit according to one of the previous claims, characterized in that it comprises a form for evaluating the obtained measurement results.

18. Diagnostic kit according to one of the previous claims, characterized in that it comprises a microarray (DNA chip) in which case the array has a number of wells (fields), separated from one another, and in at least one of the wells of the microarray an oligonucleotide is arranged which hybridizes with the looked for DNA segmcnt.

19. Diagnostic kit according to the previous claim, characterized in that in at least one other wcll of the microarray another oligonucleotide is arranged and the sequcncc of the oligonucleotide which is arranged in at least one well differs from the sequence of the other oligonucleotide.

20. Diagnostic kit according to one of the two previous claims, characterized in that in at lcast two wells an oligonucleotide is in each case arranged in which case the oligonucleotides arranged in different wells in each case hybridize with various, looked for DNA segments.

21. Microarray, for example, DNA chip, with an arrangement of several wells (fields), separated from one another, characterized in that in at least one well of the microarray an oligonucleotide is arranged which hybridizes with a DNA segment which is part of the human RhD gene.

22. Microarray according to claim 21, characterized in that in at least six wells in each case various oligonucleotides are arranged which hybridize with in each case six different DNA segments of the human RhD gene.

23. Microarray according to claim 21 or 22, characterized in that the DNA segments are segments of the coding subranges (exons) 3, 4, 5, 6, 7 and/or 9.

24. Method for determining the rhesus factor of a human characterized in that the allelic variability and/or the prcsence of the RhD gene is/are detected in a blood sample and therefrom the rhesus type and/or the rhesus sub-type of the human is determined.

25. Method according to the previous claim, characterized in that the presence of one or several of the exons 4 to 7 and/or 9 of the RhD gene is determined.

26. Method according to claim 24 or 25, characterized in that the determination of the allelic variability and/or of the presence of the RhD gene take(s) place by means of a microarray according to onc of the claims 18 to 23.

27. Method according to one of the claims 24 to 26, characterized in that the determination takes place by way of a blood sample of the person himself.

28. Method according to one of the claims 24 or 26, characterized in that the rhesus factor of a fetus is determined by analyzing a maternal blood sample.

29. Method according to claim 28, characterized in that from the maternal blood sample a fraction is separated which essentially contains fetal, nucleus-containing erythrocytes and the rhesus factor is determined by way of this fraction.

30. Method according to claim 28 or 29, characterized in that the DNA-containing constituents of thc maternal blood sample first are concentrated and subsequently this fraction is analyzed.

31. Method according to claim 30, characterized in that for concentrating the DNA-containing constituents a blood sedimentation is carried out.

32. Method according to claim 30 or 31, characterized in that for concentrating the DNA containing constituents a lysis of therein contained nucleated fetal erythrocytes is carried out and subsequently the cell-free present DNA is centrifuged off and is recovered for thc further diagnosis.

33. Method according to claim 28, characterized in that for determining the rhesus factor of the fetus thc cell-free fetal DNA in the blood plasma of the maternal blood sample is analyzed.

34. Method according to one of the claims 24 to 33, characterized in that from the blood sample or the fraction the DNA is isolated and is at least to some extent increased and subsequently the allelic variability or the presence or lack of the RhD gene is determined.

35. Method according to the previous claim, characterized in that the DNA is increased through polymerase chain reaction (PCR).

36. Method according to one of the two previous claims, characterized in that the increased DNA is digested by means of suitable restriction enzymes and based on the produced DNA fragments, the allelic variability or the presence and/or error (sic) of the RhD gene is determined.

37. Method according to one of the claims 24 to 36, characterized in that for the increase of the DNA one or several oligonucleotide pairs is/are used which have the following sequences:
TCGGTGCTGATCTCAGTGGA and ACTGATGACCATCCTCATGT
or
CACATGAACATGATGCACA and CAAACTGGGTATCGTTGCTG
or
GTGGATGTTCTGGCCAAGTT and CACCTTGCTGATCTTACC
or
GTGGCTGGGCTGATCTACG and TGTCTAGTTTCTTACCGGCAGT
or
AGCTCCATCATGGGCTACAA and ATTGCCGGCTCCGACGGTATC
or

AACAGGTTTGCTCCTAAATATT and
AAACTTGGTCATCAAAATATTTAACCT

38. Method according to claim 37, characterized in that the oligonucleotides with the sequence
GTGGATGTTCTGGCCAAGTT or AGCTCCATCATGGGCTACAA
are labeled with the fluorophore carboxyfluorescein.

39. Method according to claim 37, characterized in that the oligonucleotides with the sequence
CACATGAACATGATGCACA or AACAGGTTTGCTCCTAAATATT
are labeled with the fluorophore 4, 7, 2', 4', 5'7' –hexachloro-6-carboxyfluorescein.

40. Method according to claim 37, characterized in that the oligonucleotides with the sequence
TCGGTGCTGATCTCAGTGGA or GTGGCTGGGCTGATCTACG
are labeled with the fluorophore NED™ of the firm PE Biosystems.

41. Method according to one of the claims 38 to 40, characterized in that for the detection of the amplified DNA the fluorescent radiation emitted by the amplified DNA is determined.

42. Use of a method and/or a diagnostic kit according to one of the previous claims for the prenatal determination of the rhesus factor of a human fetus.

In addition 5 pages of drawings